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Enhancement of Secretion of Human Procollagen I in Mouse HSP47-Expressing Insect Cells

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We previously demonstrated that insect cells were able to synthesize recombinant human procollagen I as triple-helical heterotrimers when transfected with cDNAs of both proα1(I) and proα2(I) chains. However, most of the heterotrimers were retained within the cells, unlike in the case of mammalian cells [Tomita, M., Kitajima, T., and Yoshizato, K. (1997) *J. Biochem.* 1061-1069]. In an attempt to improve the secretion of the heterotrimers, we introduced the putative collagen-specific chaperone HSP47 into this insect expression model. Mouse HSP47 produced by the insect cells bound intracellularly to both human proα1(I) and proα2(I) chains and enhanced the secretion of procollagen I heterotrimers. HSP47 was also coexpressed with either proα1(I) chains or proα2(I) chains, which showed that it enhanced the secretion of the former but not the latter. This selective effect of HSP47 was similarly observed in the cells treated with inhibitors of procollagen triple helix formation, indicating that HSP47 can also accelerate the secretion of non-helical procollagens. HSP47 did not change the intracellular solubility of proα1(I) and proα2(I) chains in 1% NP-40, eliminating the possibility that it prevents proα chains from aggregating into insoluble forms within the insect cells. We concluded that HSP47 can play a role in the secretion of α1(I)-procollagen chains in the insect cell model. The present study also demonstrated the dissimilarity in the mechanism of folding and secretion of the expressed procollagen I between the insect and mammalian cells.

Key words: baculovirus expression system, collagen secretion, HSP47, recombinant collagen, type I procollagen.

Procollagen I, a precursor of type I collagen, is a heterotrimeric molecule composed of two proα1(I) chains and one proα2(I) chain, and its biosynthesis is a complex process, which has been studied mostly in vertebrate cells. The individual proα chains are translated from their mRNAs on polyribosomes bound to the endoplasmic reticulum (ER) and inserted cotranslationally into the lumen of the ER. Hydroxylation of some proline and lysine residues, and subsequent glycosylation of some hydroxylysines take place as cotranslational processes. After the completion of chain elongation, C-propeptides associate and form interchain disulfide bonded trimers, and then triple helix formation proceeds in the C- to N-terminal direction (for review, see Refs. *1* and *2*). Once folded, procollagens are readily secreted through the Golgi complex *en route* to the extracellular space, in the same way as other secretory proteins. Triple helix formation is a prerequisite for the secretion of procollagen at the maximum rate (*3*). If the helix formation is prevented by inhibiting prolyl hydroxylation, non-helical chains accumulate within the ER lumen and are then secreted at a slower rate (*4, 5*).

It has been suggested that proteins termed "molecular chaperones" and "folding enzymes" participate in regulating the folding and secretion of procollagen I (*6, 7*). Of these proteins, HSP47 is characteristic in having a substrate specificity for procollagens including types I-V (*8*). This protein was first identified in chick embryo fibroblasts (*9*) and later found in a variety of collagen-synthesizing cells of vertebrates (*10*). The expression of HSP47 is closely correlated with that of procollagens. For example, when chick embryo fibroblasts are transformed with Rous sarcoma viruses, the synthesis of both procollagen I and HSP47 is simultaneously decreased (*11*). Pulse-chase experiments combined with cross-linking and immunoprecipitation have revealed the intracellular association of HSP47 with procollagen I. Immediately after proα chains enter the ER lumen, HSP47 associates with these nascent chains. This association continues until the proα chains form triple helices, and HSP47 dissociates from the procollagens after they are exported from the ER (*12, 13*). When the triple helix formation of proα chains and their subsequent transport from the ER were blocked by an inhibitor of prolyl hydroxylation, HSP47 was stably asso-

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ciated with non-helical proα chains within the ER (*6, 12*). Thus, HSP47 is evidently involved in the folding and/or secretion of procollagen during its biosynthesis. However, the exact role of HSP47 in procollagen synthesis has not been established.

We previously described the production of the human procollagen I (*14*) and III (*15*) in a baculovirus expression system, using Sf9 insect cells as a host which possessed a certain level of prolyl hydroxylase activity. Procollagen I was produced as triple-helical heterotrimers composed of two proα1(I) chains and one proα2(I) chain by the co-expression of both chains, and as proα1(I)-homotrimers by the expression of proα1(I) chains alone. Procollagen III was synthesized as triple-helical homotrimers. However, these triple-helical procollagens had slightly lower melting temperatures than procollagens synthesized by human cells, due to the under-hydroxylation of proline residues, and they were secreted at much lower rates than in mammalian cells. The poor secretion of triple-helical procollagen III from the insect cells was also observed by Lamberg *et al.* even when the cDNAs for α and β subunits of prolyl hydroxylase were supplemented to achieve the maximum hydroxylation (*16*). This suggests that the poor secretion is not due to the insufficient prolyl hydroxylation. Several other factors such as chaperones and folding enzymes, including HSP47, BiP, GRP94, PDI, and cyclophilin B, have been suggested to regulate the process of folding and secretion of procollagens (*6, 7*). These proteins might be absent, or present in insufficient concentrations in the insect cells, or not be functional for mammalian procollagens.

Expression of the genes of human procollagen in the insect cells is clearly not the natural one, but an artificially designed expression in a heterogeneously recombined model system. However, we considered that this heterogenous expression model has an advantage in searching for the factor(s) regulating the procollagen-secretion in human cells, because the secretion of triple-helical procollagen molecules is severely suppressed in this model. This suppression is probably due either to the presence of inhibitory factors in the insect cells which are not present in human cells or to the lack of necessary factors in the insect cells. It has been suggested that HSP47 is involved in the secretion of procollagens in mammalian cells (*7, 10*). It is not known if HSP47 or its homologue is present in the insect cells. We considered that HSP47 is one of the factors lacking in the insect cells.

In the present study, we introduced the human HSP47-cDNA into the expression system together with proα1(I)- and/or proα2(I)-cDNAs, and analyzed the effect of HSP47 on the procollagen-secretion. These coexpression experiments showed that HSP47 significantly and selectively improved the secretion of procollagen I heterotrimers and proα1(I)-homotrimers, but not that of proα2(I) chains. However, the fraction of the heterotrimers in secreted procollagens was very low, indicating that other factors need to be incorporated into this insect model to optimize the folding and secretion of heterotrimers of human procollagens. We showed that the insect expression system for mammalian procollagen genes is useful for investigating the role of factors involved in the process of procollagen secretion.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses—Recombinant baculoviruses (Ac1A1/MP and Ac1A2/MP) for expressing human proα1(I) and proα2(I) chains under the control of the basic protein promoter were described previously (*14*). Recombinant viruses for expressing mouse HSP47 under the control of the same promoter were prepared as follows. The pMH47a plasmid (*17*) carrying a mouse HSP47-cDNA was digested with *Hin*dIII and *Eco*RI. The released cDNA fragment containing 147 bp of the 5′-untranslated region, the whole coding region, and 141 bp of the 3′-untranslated region was blunt-ended with T4 DNA polymerase and inserted into the transfer vector pAcMP3 (PharMingen) at the blunt-ended *Bam*HI site, downstream of the basic protein promoter. The recombinant transfer vector containing the cDNA in the correct transcriptional orientation was cotransfected into *Spodoptera frugiperda* Sf9 insect cells with a linearized *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (PharMingen) using a Lipofectin reagent (Gibco/BRL). The resulting recombinant baculoviruses AcMH47 were plaque-purified and amplified by repeated infection to Sf9 cells as described previously (*18*). Negative control viruses (AcMP) containing no inserts were prepared by cotransfection of pAcMP3 vectors and AcNPV DNA into Sf9 cells as above.

Expression and Radiolabeling of Recombinant Proteins—Sf9 cells were cultured as monolayers at 28°C in Grace's insect medium (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS). For the production of recombinant proteins, these cells were replated at a density of 10^6 cells/35-mm diameter dish and infected with each of the recombinant viruses prepared as above at a multiplicity of infection (m.o.i.) of 10. The same m.o.i. was applied to each of viruses when the cells were coinfected with two or three different viruses. At 24 h post-infection, the cells were preincubated for 1 h in proline-free Grace's medium (Sanko Jun-yaku) containing 50 μg/ml ascorbic acid (Gibco/BRL) and 10% FBS which had been dialyzed against phosphate-buffered saline (PBS), then labeled with 37 kBq/ml of [*U*-^{14}C]proline (Amersham) for 4 h. In some cases, 0.3 mM of α,α'-dipyridyl (Nacalai Tesque) or 200 μg/ml of *cis*-4-hydroxyproline (Nacalai Tesque) was added to the medium during the preincubation and labeling periods. For pulse-chase analysis, the infected cells were labeled with 74 kBq/ml of [^{14}C]proline for 10 min and chased up to 120 min in the presence of 50 mM proline and 10 μg/ml cycloheximide (Sigma). In this case, the cells were cultured in suspension according to Jarvis and Summers (*19*). The labeled cells were harvested and washed twice with PBS. Proteins in the culture medium were precipitated with 33% saturated $(NH_4)_2SO_4$ as described previously (*14*).

Gelatin Binding Assay of Recombinant Mouse HSP-47—The pellets of the labeled cells were lysed on ice for 15 min with a buffer consisting of 50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF, and 1 mM *N*-ethylmaleimide. The lysate was clarified by centrifugation. Aliquots of the resulting supernatants were mixed with gelatin-Sepharose 4B (Pharmacia) and incubated at 4°C for 2 h. The gelatin-Sepharose beads were then collected by centrifugation and washed three times with the

lysis buffer. Gelatin-bound proteins were eluted from the beads by boiling in SDS/PAGE sample buffer for 5 min, then analyzed by SDS/PAGE as described below.

Crosslinking and Immunoprecipitation—The labeled cells were resuspended in PBS and treated with a chemical cross-linker, 2 mM dithio*bis* (succinimidylpropionate) (DSP, Pierce), according to Nakai *et al.* (*6*). The cells were washed with 2 mM glycine in PBS, then with PBS, and lysed with the lysis buffer. After centrifugation, aliquots of the supernatants were mixed with anti-HSP47 antiserum (*12*) and incubated for 2 h at 4°C. Protein A-Sepharose beads (Sigma) were added to the mixtures, which were incubated for an additional 1 h at 4°C with constant shaking. The beads were washed five times with 50 mM Tris/HCl, pH 8.0, 0.4 M NaCl, and 5 mM EDTA, and once with 10 mM Tris/HCl, pH 6.8. The washed beads were resuspended in SDS/PAGE sample buffer and boiled for 5 min to release immunoprecipitates. Protein A-Sepharose beads were removed by centrifugation, and the supernatants were analyzed by SDS/PAGE.

Analysis of Recombinant Human Proα Chains—The pellets of labeled cells and the $(NH_4)_2SO_4$-precipitates of the culture medium were dissolved in SDS/PAGE sample buffer and subjected to SDS/PAGE to analyze the secretion of recombinant proα chains. For pepsin digestion, the cell pellets and the precipitates of the medium were suspended in 0.5 M acetic acid containing 1% Triton X-100 and treated with 100 μg/ml of the enzyme at 4°C for 16 h. The reaction was stopped by neutralization with NaOH and addition of SDS/PAGE sample buffer. For the digestion with a mixture of trypsin and chymotrypsin (both from Sigma), the cell pellets were dissolved in 0.4 M NaCl, 1% Triton X-100, and 0.1 M Tris/HCl, pH 7.4, incubated at 28°C for 5 min, then digested at 20°C for 2 min with the enzymes at the concentration of 100 μg/ml and 250 μg/ml, respectively. The digests were immediately mixed with 0.5 mg/ml of soybean trypsin inhibitor (Sigma) and an equal volume of two times concentrated boiling SDS/PAGE sample buffer. The solubility of proα chains in the cells was determined by dissolving the cell pellets with the lysis buffer as described above. After centrifugation, the clarified supernatants and insoluble pellets were separately treated with SDS/PAGE sample buffer for electrophoresis to compare soluble and insoluble proα chains.

Electrophoresis was performed on SDS-polyacrylamide gels according to Laemmli (*20*). The gels were fixed, dried, and exposed to Kodak X-Omat AR films or BAS2000 imaging plates (Fuji Film). Relative radioactivity of protein bands was quantified using a Bio-Image Analyzer BAS2000 (Fuji Film) as described previously (*14*).

RESULTS

Expression of Mouse HSP47 in Insect Cells—We previously showed that full-length human proα1(I) and proα2(I) chains were efficiently synthesized in Sf9 insect cells under the control of the basic protein promoter (*14, 21*). This promoter was also employed in the present study. The recombinant baculoviruses AcMH47 containing the mouse HSP47-cDNA and the control viruses AcMP containing no inserts were constructed and infected into Sf9 cells. The cells were labeled with [^{14}C]proline for 4 h at 1 day post-infection, and proteins in the cells were analyzed by SDS/PAGE under reducing condition. A protein of approximately 47 kDa was detected in AcMH47-infected cells but not in AcMP-infected cells (Fig. 1A). This recombinant product possessed gelatin-binding activity (Fig. 1B), demonstrating that functional HSP47 was produced by Sf9 insect cells infected with AcMH47 viruses. However, the gelatin-bound HSP47 was about 10% of NP-40-solubilized HSP47. This may be explained by the previous finding that the binding between HSP47 and collagens was specific but of relatively low affinity (*8*).

Association of HSP47 with Procollagen in Insect Cells—The intracellular association of the expressed HSP47 with proα chains was determined by immunoprecipitation analysis. Sf9 cells were infected with either Ac1A1/MP, Ac1A2/MP, or AcMH47, or coinfected with two or three of these viruses, each at m.o.i. 10. The infected cells were treated with a chemical cross-linker (DSP), and subjected to immunoprecipitation with anti-HSP47 antibodies. Two proteins were precipitated with the antibody in addition to HSP47 from the cells infected with AcMH47 alone (Fig. 2, lane 1, see asterisks). These proteins were not precipitated from the lysates of the cells infected with Ac1A1/MP or Ac1A2/MP alone (Fig. 2, lanes 2 and 3), suggesting that they were associated with HSP47 in the cells. The proteins have not been further characterized at present. In addition to these insect proteins, recombinant proα1(I) or proα2(I) chains were coprecipitated with HSP47 when the cells were coinfected with AcMH47 and either of the procollagen viruses (Fig. 2, lanes 4 and 5). The intracellular association of HSP47 with both proα chains was also observed in the cells coinfected with three recombinant viruses (Fig. 2, lane 6). In both cases, the amount of precipitated proα1(I) chains was less than that of proα2(I) chains, although we confirmed that proα1(I) and proα2(I) chains were expressed at similar levels when each virus was infected into cells

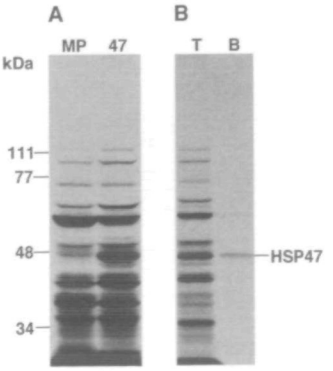

Fig. 1. **Expression of mouse HSP47 in insect cells.** (A) Synthesis of HSP47. Sf9 cells were infected with control viruses (AcMP) or recombinant viruses carrying mouse HSP47-cDNA (AcMH47). The cells were labeled with 37 kBq/ml of [^{14}C]proline for 4 h at 1 day post-infection. One million labeled cells from controls (MP) and experiments (47) were dissolved in SDS/PAGE sample buffer and subjected to 8% SDS/PAGE. Radioactive proteins on the gels were visualized by autoradiography. (B) Gelatin binding of recombinant HSP47. Proteins were extracted from 10^8 AcMH47-infected cells with lysis buffer containing 1% NP-40 (T). The extract was mixed with gelatin-Sepharose beads and incubated at 4°C for 2 h. After washing, the beads were boiled in SDS/PAGE sample buffer to elute gelatin-bound proteins (B). The proteins were electrophoresed and visualized by autoradiography as in (A). The arabic numerals at the left side of the gels are molecular masses in kDa.

at the same m.o.i. (data not shown, see Ref. *14*). This may be explained by the result that proα1(I) chains were more difficult to extract from the cells treated with the cross-linker than proα2(I) chains (data not shown).

We tested the possibility that the extent of hydroxylation of proline residues of procollagens affects the binding of HSP47 to them. Virus-infected cells were cultured in the presence of α,α'-dipyridyl, an inhibitor of prolyl and lysyl hydroxylases (*22*), then subjected to the immunoprecipitation as above. Proα1(I) and proα2(I) chains were coprecipitated with HSP47 to a similar extent to that in the untreated cells (Fig. 2, lanes 7-9), clearly indicating that HSP47 does not discriminate between the unhydroxylated and hydroxylated chains when it associates with them.

Effect of HSP47 on the Secretion of Procollagen I Heterotrimers—Our previous study showed that pepsin-resistant procollagen I heterotrimers composed of two proα1(I) and one proα2(I) chains can be produced by Sf9 cells infected with both Ac1A1/MP and Ac1A2/MP (*14*). However, most of the heterotrimers remain within the cells, and only a small fraction is secreted into culture medium (*14*). The present study was performed to determine if HSP47 improves the secretion of procollagen I heterotrimers. To this end, Sf9 cells were triply infected with Ac1A1/MP, Ac1A2/MP, and AcMH47. As a negative control, Sf9 cells were infected with Ac1A1/MP, Ac1A2/MP, and AcMP instead of AcMH47. The expression levels of proα chains were equalized between the HSP47-expressing cells and the non-expressing cells. The infected cells were labeled with [^{14}C]proline for 4 h, and proteins in the cell and culture medium were subjected to SDS/PAGE. The ratio of secreted proα chains to total proα chains was determined by measuring the relative radioactivity of each proα chain present in the cell and culture medium.

In the absence of HSP47, the ratio of secreted proα1(I) chains was 2.5%, which was considerably lower than that (20%) of secreted proα2(I) chains (Fig. 3A, lanes 1 and 2, and Fig. 3B). Coexpression of HSP47 increased the ratio of secreted proα1(I) chains approximately three times, but had no significant effect on the secretion of proα2(I) chains (Fig. 3A, lanes 3 and 4, and Fig. 3B). The virus-infected Sf9 cells were labeled with [^{14}C]proline for 10 min and chased in the presence of excess unlabeled proline. The pulse-labeled proα1(I) chains remained undegraded in the cells for at least 2 h (data not shown).

As reported previously, these proα1(I) chains take part in heterotrimer formation, and excess proα2(I) chains exist as monomers or as unstable homotrimers in Sf9 cells (*14*). Therefore, the increase of proα1(I)-secretion in the HSP47-synthesizing cells suggests that HSP47 enhanced the secretion of heterotrimers. It should be noted that the vast majority of the chains still remained within the cells even in the presence of HSP47 (Fig. 3A, lane 3 and lane 4). Our previous study showed that about 20% of the chains remaining in the cells are in the form of pepsin-resistant heterotrimers (*14*). To confirm the formation of hetero-

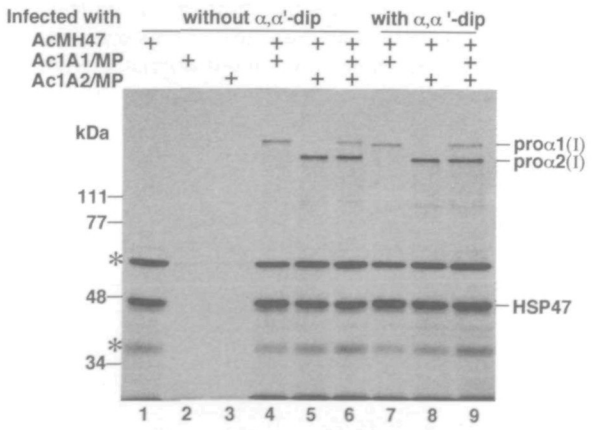

Fig. 2. **Intracellular association of HSP47 with proα chains.** Sf9 cells were singly infected with AcMH47 or proα chain-recombinant viruses (Ac1A1/MP or Ac1A2/MP) (lanes 1-3), or coinfected with two or three of these viruses (lanes 4-9) as indicated at the top left of the figure. At 1 day post-infection, the infected cells were labeled with 37 kBq/ml of [^{14}C]proline for 4 h in the absence (lanes 1-6) or presence (lanes 7-9) of 3 mM α,α'-dipyridyl (α,α'-dip). The infected cells were treated with 2 mM DSP, washed with 2 mM glycine in PBS, and extracted with lysis buffer containing 1% NP-40. The extracts were incubated for 2 h at 4 C with anti-HSP47 antiserum. Immuno-complexes were precipitated by adding protein A-Sepharose beads and analyzed by 8% SDS/PAGE. Visualization was achieved by autoradiography. Asterisks indicate proteins coprecipitated with anti-HSP47 antiserum. Pluses represent that the cells were infected with the indicated viruses. The bands corresponding to proα1(I), proα2(I), and HSP47 are indicated by these abbreviations at the right side of the gel. The numerals at the left side of the gel are molecular masses in kDa. Precipitated proα1(I) and proα2(I) chains were slightly smaller than those synthesized by untreated cells due to the inhibition of hydroxylation by α,α'-dipyridyl.

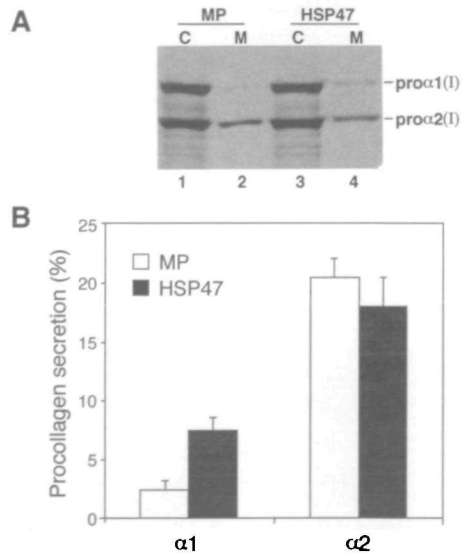

Fig. 3. **Effect of HSP47 on the secretion of procollagen I heterotrimers.** (A) Synthesis and secretion of proα chains. Sf9 cells were infected with AcMP (MP) or AcMH47 (HSP47) in addition to Ac1A1/MP and Ac1A2/MP. The infected cells were labeled with 37 kBq/ml of [^{14}C]proline for 4 h. The labeled cells were harvested and dissolved in SDS/PAGE sample buffer (C). Proα chains in the culture medium were precipitated with 33% $(NH_4)_2SO_4$ at 4°C and collected by centrifugation (M). Protein samples from equal numbers of cells were electrophoresed on 6% polyacrylamide gels, then the gels were dried and exposed to X-ray films. (B) The dried gels were exposed to BAS2000 imaging plates, and the relative radioactivity of the bands of proα chains was quantified by a BAS2000 image analyzer. Procollagen secretion (%) represents the amount of proα1(I) (α1) or proα2(I) chains (α2) in the medium as a percentage of the sum of the cell and medium fractions. Bars indicate the standard deviation from the mean of triplicate determinations.

trimers, the samples were treated with pepsin before the analysis by SDS/PAGE. Pepsin-resistant α1(I) and α2(I) chains were detected in the cell fraction (Fig. 4, lanes 2 and 4). The existence of the pepsin-resistant α2(I) chains indicated the formation of heterotrimers. The ratio of pepsin resistant α1(I) to α2(I) chains was above two, indicating that proα1(I)-homotrimers were also formed in the cells in the presence of HSP47. In addition, a pepsin-resistant band was observed between α1(I) and α2(I) chains. This band could be derived from a partially digested product of α1(I) chains, although it was not clear whether this band was derived from $[\alpha 1(I)]_3$-homotrimers or $[\alpha 1(I)]_2\alpha 2(I)$-heterotrimers. HSP47 did not show any effect on the amount of pepsin-resistant chains or on the ratio of α1(I) to α2(I) chains in the cells (Fig. 4, lanes 1-4), suggesting that HSP47 did not affect the formation of triple helices in the insect cells. On the other hand, HSP47 significantly increased the secretion of pepsin-resistant α1(I) chains into the medium (Fig. 4, lanes 5-8). This increase was accompanied by the increase of pepsin resistant α2(I) chains, indicating that HSP47 facilitated the secretion of heterotrimers. However, comparison of the intensity of bands of proα1(I) (lane 7) and α1(I) (lane 8) showed that the fraction of proα1 chains that participated in the formation of stable triple helices was still small.

Effect of HSP47 on the Secretion of Proα1(I)-Homotrimers—As described above, a fraction of proα1(I) chains is present in the form of proα1(I)-homotrimers. To investigate whether HSP47 enhances the secretion of proα1(I)-homotrimers, Sf9 cells were infected together with AcMH47 and proα1(I) chain-viruses (Fig. 5). The effect of HSP47 on the secretion of proα2(I) chains was also determined by coinfecting the cells with AcMH47 and proα2(I) viruses. The ratio of secreted proα1(I) chains was significantly increased by coexpression of HSP47, whereas that of proα2(I) chains was not affected.

The observed enhancement of secretion of proα1-containing triple-helical procollagens (homo- and heterotrimers) by HSP47 could be explained if HSP47 increases the intracellular solubility of the procollagens. This possibility was tested. Sf9 cells were infected with Ac1A1/MP or Ac1A2/MP together with or without AcMH47. These cells were incubated with [^{14}C]proline for 4 h, then lysed with the lysis buffer containing 1% NP-40 and 0.15 M NaCl. The ratio of solubilized proα chains to total intracellular chains was measured. In the absence of HSP47, 50-60% of both proα chains were solubilized. No significant difference in the intracellular solubility was detected between proα1(I) chains and proα2(I) chains. Moreover, solubilities of proα1(I) and proα2(I) chains were not affected by the expression of HSP47. Essentially the same result was obtained when the cells were lysed with the buffer not containing NaCl (data not shown).

Effect of HSP47 on the Secretion of Non-Helical α1(I) Procollagens—As shown in Figs. 3 and 4, HSP47 promoted the secretion of α1(I)-containing procollagens about 3 times. These procollagens included homotrimers (proα1-trimers) and heterotrimers (proα1/α2-trimers) as minor constituents. Figure 4 shows that the major constituent of secreted proα1(I) procollagens in the presence of HSP47 was pepsin-digestible and, therefore, non-helical procollagens. To examine the effect of HSP47 on these major procollagens, we investigated if HSP47 actually stimulates the secretion of non-helical procollagen I. The cells were infected with AcMP or AcMH47 in addition to Ac1A1/MP and labeled with [^{14}C]proline in the presence of an inhibitor of triple helix formation, α,α'-dipyridyl (*23, 24*) (Fig. 6). The treatment with 0.3 mM inhibitor enhanced the secretion of proα1(I) chains whether HSP47 was present or not (Fig. 6B). The treatment also enhanced the intracellular solubility of both chains to 85-87% (data not shown). The enhancement by α,α'-dipyridyl may be partly due to this increased solubility.

Digestion of proα chains with a mixture of trypsin and chymotrypsin at 28°C confirmed that proα1(I) chains synthesized in the presence of α,α'-dipyridyl exist as non-helical molecules (Fig. 6A, lanes 1-4). The secretion of these non-helical proα1(I) chains was enhanced by coexpression of HSP47 (Fig. 6B). Solubilities of unhydroxy-

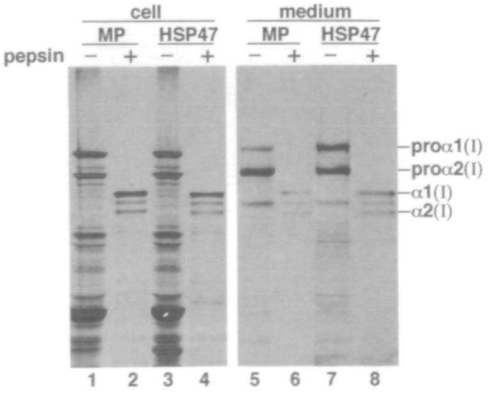

Fig. 4. **Pepsin treatment of procollagen I heterotrimers.** Sf9 cells were infected with AcMP (MP) or AcMH47 (HSP47) together with Ac1A1/MP and Ac1A2/MP and labelled as in Fig. 3. The cell pellets (lanes 1-4) and $(NH_4)_2SO_4$-precipitates of the culture medium (lanes 5-8) were suspended in 0.5 M acetic acid containing 1% Triton X-100 and treated with (+) or without (−) 100 μg/ml pepsin at 4°C for 16 h. The samples were analyzed by 6% SDS/PAGE. The gel containing lanes 5-8 was exposed to X-ray films for five times longer than the gel containing lanes 1-4. The band observed between α1(I) and α2(I) chains may be a partially digested product of proα1(I) chains.

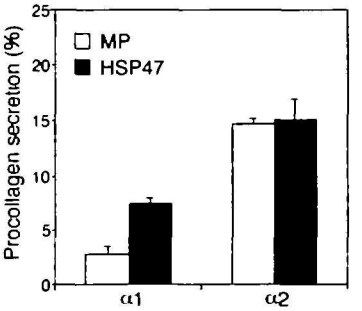

Fig. 5. **Effect of HSP47 on the secretion of proα1(I) chains.** Sf9 cells were infected with AcMP (MP) or AcMH47 (HSP47) in addition to Ac1A1/MP (α1) or Ac1A2/MP (α2). The infected cells were labelled with 37 kBq/ml of [^{14}C]proline for 4 h. Protein samples were prepared from the cell and medium fractions as in Fig. 3 and electrophoresed in polyacrylamide gels. The gels were analyzed with the image analyzer, and the percentage of procollagen secretion was calculated. Bars indicate the standard deviation from the mean of triplicate determinations.

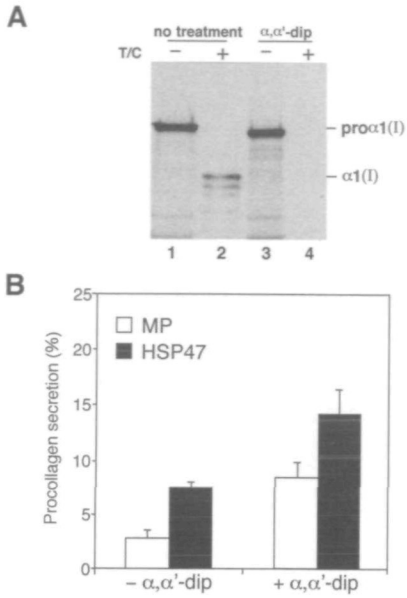

Fig. 6. **Effect of HSP47 on the secretion of non-helical proα1(I) chains.** (A) Inhibition of triple helix formation of procollagens by α,α′-dipyridyl. Sf9 cells were infected with Ac1A1/MP and labeled with 37 kBq/ml of [^{14}C]proline for 4 h in the absence (no treatment, lanes 1 and 2) or presence of 0.3 mM α,α′-dipyridyl (α,α-dip, lanes 3 and 4). The labeled cells were harvested and lysed with buffer containing 1% Triton X-100. After incubation at 28°C for 5 min, the samples were treated with (+) or without (−) a mixture of 100 μg/ml trypsin and 250 μg/ml chymotrypsin (T/C) at 20°C for 2 min. The digests were analyzed by 6% SDS/PAGE and autoradiography. (B) Effect of HSP47 on the secretion of α1(I)-procollagens. Sf9 cells were infected with AcMP (MP) or AcMH47 (HSP47) in addition to Ac1A1/MP and labeled with [^{14}C]proline for 4 h in the presence of 0.3 mM α,α′-dipyridyl. After electrophoresis of both cell and medium fractions, the percentage of secreted proα1(I) chains in the total synthesized chains was determined as in Fig. 3. Bars indicate the standard deviation from the mean of triplicate determinations.

lated proα1(I) chains in the lysis buffer were unaltered by HSP47, as described above. A similar enhancement of the secretion by HSP47 was observed in the presence of 200 μg/ml *cis*-4-hydroxyproline, another inhibitor of the formation of helical conformation (*25*) (data not shown). Therefore, it was concluded that HSP47 enhanced the secretion of non-helical proα1(I) chains.

DISCUSSION

In mammals, chains of proα1(I) and proα2(I) are generally assembled into heterotrimers of [proα1(I)]$_2$[proα2(I)], which are rapidly secreted from the cells. Proα1(I) chains can be also secreted as homotrimers of [proα1(I)]$_3$ when the cells synthesize only proα1(I) chains (*26, 27*) or when the cells synthesize mutated proα2(I) chains that cannot associate with proα1(I) chains (*28-30*). In contrast, proα2(I) chains are not usually secreted unless they are associated with proα1(I) chains (*31, 32*). On the other hand, our present and previous (*14*) studies showed that human proα chains were secreted in a different manner when expressed in insect cells. Most of the expressed proα1(I) chains remained within the insect cells, whether infected with Ac1A1/MP alone or together with Ac1A2/MP, and small amounts of them (less than 5%) were secreted into the culture medium. Less than 50% of the chains remaining within the cells were triple-helical procollagens, either α1-homotrimers or α1/α2-heterotrimers, and the remainder were non-helical chains. It should be noted that these non-helical chains remained stable and were not degraded. Triple-helical and non-helical chains were also found in similar proportions in the secreted proα1-collagens. On the other hand, about 80% of the expressed proα2(I) chains remained within the cells, and these chains were non-helical both within the cells and in the medium. They were also stable and not degraded. These results suggest that the insect cells lack factors operating in human cells to regulate the secretion of human procollagen. The present study demonstrated that one of these factors was HSP47, because coexpression of HSP47 in the insect cells improved the secretion rates of proα1(I)-homotrimers and heterotrimers. This selective action of HSP47 on proα1(I)-containing procollagens must also contribute to the proper secretion of procollagen in mammalian cells. This finding provides an example of the usefulness of the insect expression system for mammalian procollagen genes as a model to investigate the factors involved in the process of the secretion of their translates.

The present study demonstrated that HSP47 enhanced the secretion of α1(I)-procollagens, but not that of proα2(I) chains, although HSP47 bound equally to both proα1 and proα2 chains in the cells. There are several possible explanations of this selective effect of HSP47. Small fractions of proα1(I) collagen chains are triple-helical homotrimers and heterotrimers at the temperature of the culture of Sf9 cells (28°C). On the other hand, proα2(I) chains cannot form triple helices by themselves at temperatures above 20-24°C, even when their prolyl hydroxylations are normal (*33*). Therefore, the selective effect of HSP47 on proα1(I) chains might be explained by a difference in the ability to form triple helices. However, the present study ruled out this possibility. We investigated whether the triple-helical conformation is necessary for HSP47 to work. The secretion of non-helical proα1(I) chains synthesized in the presence of α,α′-dipyridyl was enhanced by coexpressed HSP47, while that of proα2(I) chains was not affected. The enhancement of the secretion of non-helical proα1(I) chains by HSP47 was also observed when the cells were treated with another inhibitor, *cis*-4-hydroxyproline. Accordingly, we concluded that the effect of HSP47 on procollagen-secretion does not depend on the triple-helicity of procollagen, which eliminates this possibility for the selective effect of HSP47.

Another possibility was that proα1(I)-containing procollagens form insoluble aggregates in the cells in the absence of HSP47, whereas proα2(I) chains do not. If HSP47 prevents this aggregation, its presence would result in the increase of secreted procollagens, as in the case of BiP-enhanced secretion of IgG from insect cells (*34*). Solubilization experiments with 1% NP-40 did not provide evidence for this possibility. No significant difference in intracellular solubility was found between proα1(I) and proα2(I) chains, and coexpression of HSP47 failed to affect the solubility of both chains.

A further possibility is that aggregates or protein complexes containing proα1(I) chains are formed by weak interactions that are susceptible to HSP47. Recently, it was reported that newly synthesized proα chains and protein

disulfide isomerase (PDI) transiently form large molecular size protein complexes with weak interactions in the ER lumen of human fibroblasts during the normal maturation pathway of procollagen (*35*). Formation and/or dissociation of such protein complexes may be mediated by HSP47. This possibility remains to be tested.

According to the current view of the protein transport, folded secretory or membrane proteins in the ER lumen are sorted and concentrated into COPI- or COPII-coated transport vesicles during their budding from the ER, then transported through the intermediate compartment to the *cis*-Golgi compartment (*36-39*). Previous studies suggested the involvement of HSP47 in this transport pathway. HSP47 associates with newly synthesized procollagen in the ER of fibroblasts and remains bound to them in the intermediate compartments. It is then dissociated from the procollagen in the *cis*-Golgi compartment, and retrogradely transported back to the ER *via* a retrieval flow with KDEL receptor (*40, 41*), which recognizes the RDEL sequence at the COOH terminus of HSP47 (*12*).

Since the KDEL sequence of other ER-resident proteins functions as the ER retention signal in insect cells, even when the cells are compromised by baculovirus infection (*42, 43*), it is likely that the expressed HSP47 shows the same localization and function in insect cells as it does in vertebrate fibroblasts. From these previous studies and our present results showing the enhanced secretion of pro α1(I)-containing procollagens by HSP47, it is conceivable that HSP47 participates in the sorting and concentration of the procollagens into transport vesicles budding from the ER in mammalian cells. HSP47 might deliver procollagen into defined regions of the ER at which the vesicle formation occurs, or mediate indirect association between COP-coats and procollagen, which would ensure the packaging of procollagen into COP-coated transport vesicles. These roles are probably irrelevant to proα2(I) chains, because the enhancement of the procollagen-secretion by HSP47 was observed for proα1(I) chains but not for proα2(I) chains. A previous study using fibroblasts from osteogenesis imperfecta patients revealed that mutated proα1(I) chains, which are unable to assemble efficiently, cannot escape from the ER but are degraded at this site. On the other hand, excess proα2(I) chains synthesized in the same cells are exported from the ER in a monomeric form and degraded in a post-ER site (*44*). These and our results thus imply that the export of proα2(I) chains from the ER may be regulated by an HSP47-independent mechanism.

It was recently reported that HSP47 delayed the secretion of procollagen III in human 293 cells cotransfected with HSP47- and procollagen III cDNAs (*45*). When procollagen III cDNA was singly transfected, procollagen III was abnormally modified, and this abnormal modification was inhibited by the coexpression of HSP47. These observations differ from our present results showing the enhancement of procollagen I secretion by HSP47. However, it is difficult to compare these two studies, because the type of procollagen cDNA and the type of cells studied were different.

HSP47 improved the secretion of triple-helical proα1-collagens to some extent. However, the overall status of the folding and secretion of procollagen I was not affected by HSP47. HSP47 did not prevent the secretion of proα2(I) chains or non-helical proα1(I) chains from insect cells. In vertebrate fibroblasts, such unfolded proα chains are selectively retained and degraded within the cells by a mechanism termed "quality control" (*4, 5, 29-32, 44, 46*). This mechanism is generally observed in the synthesis of secretory and membrane proteins (*47*) and believed to be mediated by molecular chaperones such as BiP (*48*) or calnexin (*49*). The observation that HSP47 is stably associated with procollagen when fibroblasts are treated with α,α'-dipyridyl suggests that HSP47 is involved in the quality control of procollagen (*6, 12*). However, our present results did not provide evidence for this possibility in the insect cells. BiP is another candidate for the role of quality control, because this protein preferentially associates with malfolded proα1(I) chains with C-propeptide mutations (*44, 50*). BiP might play a role in this mechanism independently of or in cooperation with HSP47.

In addition to HSP47, other ER-resident chaperones and folding enzymes are reported to be associated with procollagens in fibroblasts, which include BiP, GRP94, PDI, and cyclophilin B (*6, 7*). These proteins bind to a wide variety of secretory and membrane proteins in the ER as general chaperones or folding enzymes (*47*). On the other hand, HSP47 has been postulated to be a collagen-specific chaperone because of its substrate specificity. It is possible that HSP47 works together with these general chaperones and folding enzymes in a series of coupled or successive reactions during procollagen folding and secretion in mammalian cells. The insect system described in the present study might lack these general chaperones and folding enzymes. Alternatively, the insect cells might contain insect homologs of these proteins that cannot work on the human procollagens. The insect system is useful for investigating the expression and secretion of human procollagens, because, as we showed in this study, the insect cells require the coexpression of proteins involved in these processes of human collagen biosynthesis. It will be interesting to learn whether the coexpression of other cDNAs of proteins that are responsible for the quality control of the transport and the secretion of human procollagen will improve the process to an extent found in mammalian cells.

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